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5-HMF induces anaphylactoid reactions *in vivo* and *in vitro*

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ABSTRACT

Aim: Excessive exposure to 5-hydroxymethylfurfural (5-HMF), which is a common impurity in various sugar-containing products, induces serious side effects. Our previous study revealed that 5-HMF exerted immune sensitizing potential when injected into rodents. In this study, we explored 5-HMF mediated anaphylactoid reactions and its underlying molecular mechanisms.

Methods: We investigated anaphylactoid reactions in Brown Norway (BN) rats and Institute of Cancer Research (ICR) mice to identify 5-HMF mediated *in vivo* anaphylactoid reactions. RBL-2H3 and P815 cell degranulation models were also established, and degranulation, enzyme-linked immunosorbent, filamentous actin (F-actin) microfilament staining, and western blot assays were performed in these cells.

Results: We showed that 5-HMF induced anaphylactoid reactions by increasing blood vessel permeability in mice, and significantly elevating histamine (His) and glutathione peroxidase-1 (Gpx-1) levels in rat serum. Moreover, after incubation with 5-HMF, β-hexosaminidase (β-Hex), His, IL-4 and IL-6 levels were all significantly increased, thereby inducing cellular degranulation in RBL-2H3 and P815 cells. Finally, 5-HMF also upregulated Lyn, Syk, p38 and JNK protein phosphorylation levels.

Conclusions: Our findings suggest that 5-HMF induces anaphylactoid reactions both *in vivo* and *in vitro*, therefore 5-HMF limits in sugar-containing products should receive more regulatory attention.

1. Introduction

5-hydroxymethylfurfural (5-HMF, $C_3H_6O_3$) is a low molecular weight compound (LMWC), with a furan ring comprising the basic structure (Fig. 1). The compound is typically produced by Maillard or caramelization reactions of reducing sugars, at high temperatures [1,2]. Thus, 5-HMF is detected in sacchariferous drugs and foods undergoing thermal processing and long-term storage [3]. Several studies have shown that 5-HMF is a potentially harmful substance upon intravenous or oral exposure [4,5]. Similarly, 5-HMF at high concentrations (> 75 mg/kg) can cause skin, respiratory and mucosal irritation, including muscle and viscera damage [6,7]. Importantly, specification limits for 5-HMF in glucose injection solutions have also been listed in several pharmacopeias, *e.g.*, both the Chinese and United States editions stipulate that 5-HMF absorbance at 284 nm cannot exceed 0.32 in a 1% glucose injection solution, and 0.25 in a 0.4 % glucose injection solution, respectively [7,8], however, the potential health effects of 5-HMF have not yet been clarified. Our previous study demonstrated that LMWC 5-HMF exerted immune-sensitizing potential by generating co-stimulatory signals when injected into Balb/c mice [7]. Nevertheless, 5-HMF immunotoxicity and its underlying mechanisms require further elucidation.

Anaphylactoid reactions are hypersensitive-like pathological processes, without prior sensitization and non-immunoglobulin E (IgE)-

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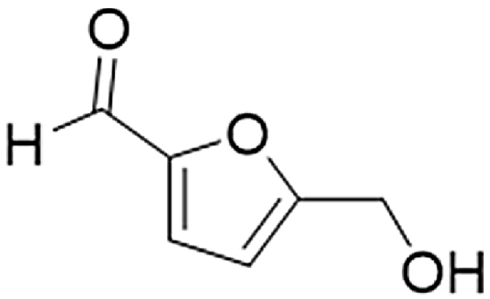

Fig. 1. 5-HMF structural information.

mediation, defined as cutaneous reactions (urticaria, pruritus, angioedema) or systemic reactions (hypotension, respiratory symptoms) [9,10]. The reaction consists of a stimulation phase, where initial antigen entry into the organism directly activates mast cell/basophil degranulation, triggers a series of biochemical events, leading to the release of active mediators and initiating symptom development [11]. Inflammatory mediators released by mast cells and basophils are responsible for the symptoms and signs of anaphylactic and anaphylactoid reactions. They differ only in the initial cause of mast cell or basophil activation [12]. Recent studies have indicated that anaphylactoid reaction incidences are much higher than IgE-mediated hypersensitivity reactions, and can be caused by many substances [13–15]. In the preclinical toxicological evaluation of a new fluorescent anti-CEA chimeric antibody SGM-101, some of the beagle dogs showed injection reaction, then resolved spontaneously, possibly due to the anaphylactoid reaction [16]. And systemic side effects induced by Iodinated contrast media may be also attributed to anaphylactoid reaction [17]. In the mechanism of digestive system diseases induced by wheat, the symptoms of non-celiac gluten sensitivity (non-autoimmune, nonallergic) are similar to those of celiac disease (an autoimmune mechanism), but the specific antibodies and intestinal lesions found in celiac disease can not be observed in the former [18]. In addition, some saccharide-containing drugs can also cause anaphylactoid reactions [19]. And it is speculated that clinical anaphylactoid reactions caused by some saccharide-containing drugs may be related to 5-HMF. Therefore, potential immunotoxicity and related mechanisms caused by anaphylactoid reactions mediated by 5-HMF-containing products cannot be ignored. To this end, we focused on 5-HMF immunotoxic mechanisms, in *in vivo* and *in vitro* models.

2. Materials and methods

2.1. Ethics statement and animals

The experimental protocols for animal use were approved by the laboratory animal welfare ethics committee of Beijing Union-Genius Pharmaceutical Technology Development Co., Ltd. (Beijing, China) (No; 0000254, August 16th, 2016). Specific pathogen free (SPF) grade Brown Norway (BN) rats (male, 6-week-old, 170 ± 20 g), and adult healthy male Institute of Cancer (ICR) mice (male, 6-week-old, 20–25 g), were purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The animal source production license number was; SCXK (Beijing) 2016−0006. Animals were permitted free access to purified water and animal diets. The ambient temperature was 23 °C ± 3 °C, relative humidity was 55 % ± 15 %, with a 12 h light/dark cycle. The animals were housed in the Animal Center of Peking Union-Genius Medical Technology Development Co. These facilities achieved accreditation (1438#) from the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International), and animals were maintained according to the 'Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

2.2. Systemic anaphylactoid reactions in ICR mice

ICR mice were randomly divided into four groups based on body weight. Animals were injected with 1) normal saline, 2) compound 48/80 (2.5 mg/kg as positive control), and 3) 5-HMF (7 mg/kg or 35 mg/kg as the low dose (5-HMF-L) and the high dose (5-HMF-H) groups, respectively) through the tail vein, in combination with 0.4 % Evans blue (n = 3 mice/group). Mice were euthanized (cervical dislocation after isoflurane inhalation anesthesia) 30 min after drug administration. Then the ears were removed. Each ear was dissected and placed into 800 μL acetone-saline (7:3) at 65 °C for 12 h. After centrifuging for 15 min (3000 rpm at 4 °C), 200 μL supernatants were added to a 96-well plate. Different concentrations of Evans blue solution were also added to generate a standard curve. Absorbance was measured at 620 nm on a microplate reader (Berthold, TriStar2S LB 942, Germany).

2.3. Systemic anaphylactoid reactions in BN rats

BN rats were randomly divided into four groups based on body weight, and injected with 1) normal saline, 2) C48/80 (5 mg/kg as positive control), and 3, 4) 5-HMF (5 mg/kg or 25 mg/kg in 5-HMF-L and 5-HMF-H groups, respectively) through the tail vein (n = 3 rats/group). Rats were anesthetized after 30 min by 1% sodium pentobarbital sodium solution, followed by blood collection from the abdominal aorta. The blood was collected into chilled blood tubes containing EDTA-K2, and centrifuged for 10 min (2500 rpm at 4 °C) to isolate serum. Glutathione peroxidase-1 (Gpx-1) and histamine (His) levels were measured by ELISA (Enzyme-linked Biotechnology, Shanghai, China) following manufacturer's instructions.

2.4. Reagents and cell culture

5-HMF was purchased from Sigma (St Louis, MO, USA; HPLC ≥ 99 %). Rat basophilic leukemia 2H3 (RBL-2H3) cells were purchased from the Cell Resource Centre of the Shanghai Institute for Biological Sciences, and cultured in modified Eagle medium (MEM, XiGong Biological Technology, Beijing, China). The culture medium was supplemented with 15 % fetal bovine serum (FBS; Gibco, Rockville, MD) and 100 U mL^{-1} penicillin/streptomycin (Sigma). Cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere. P815 cells (mastocytoma cells of the DBA/2 mouse) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in 1640 medium (1640, XiGong Biological Technology) with 10 % fetal bovine serum (FBS; Gibco, Rockville, MD). The culture medium was supplemented with 10 % fetal bovine serum (FBS; Gibco, Rockville, MD) and 100 U mL^{-1} penicillin/streptomycin (Sigma). Cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere. Experiments were performed using logarithmic growth cells.

2.5. Cell viability assay

RBL-2H3 and P815 cells were seeded in 96-well plates at 1×10^5 cells/mL. Cell viability was assessed by MTT assay, and was performed according to previous methods [20]. IC_{50} values of 5-HMF in both cell lines were also examined after 24 h incubation. To determine the non-lethal toxicity of 5-HMF, cell survival rates (cells treated with an IC_{50} and 0.1 IC_{50} dose of 5-HMF) at different time points (0–24 h) were also determined by MTT assay.

2.6. β-hexosaminidase (β-hex) and His assay

RBL-2H3 and P815 cells were seeded in 24-well plates at 1×10^5 cells/mL. C48/80 at 15 μg/mL (positive control) or different concentrations of 5-HMF (0.1 IC_{50} and IC_{50}) were then administered to cells. After cell stimulation for 1 h, supernatants were collected for β-Hex and His assay, which were performed according to previous methods [20,

21]. β-Hex and His release rates were calculated as percentages, when compared to the lysed group: *i.e.*, release ratio (%) = (T—B)/(ZB) × 100—%, where T, B and Z represented the OD of cells treated with drugs, cells without drugs, and cells lysed by 1% Triton X-100 (Sigma, MO, USA), respectively.

2.7. IL-4 and IL-6 measurements

RBL-2H3 and P815 cells were seeded and cultured overnight in 24-well plates at 1×10^5 cells/mL. Plates were then washed twice in advanced Tyrode's solution to remove residual medium. C48/80 (15 μg/mL) or different concentrations of 5-HMF (0.1 IC_{50} and IC_{50}) were administered to cells. After cell stimulation for 1 h, supernatants were collected and IL-4 and IL-6 levels were measured by ELISA (Jingmei Biotechnology, Yancheng, China) following manufacturer's instructions.

2.8. Toluidine blue staining

RBL-2H3 and P815 cells were seeded and cultured overnight in 6-well plates at 1×10^5 cells/mL on cell climbing slices. After cells were treated with different 5-HMF concentrations (0.1 IC_{50} and IC_{50}) or C48/80 (15 μg/mL) for 1 h, slices were placed in 95 % ethanol. Then toluidine blue staining solution (Solarbio, Beijing, China) was added, and cell morphology observed under a microscope (CARL ZEISS-Axio Ver AI, Germany).

2.9. F-actin microfilament staining

Phalloidin is a cyclic heptapeptide toxin derived from *Amanita phalloides* that binds specifically to F-actin [22]. RBL-2H3 and P815 cells were seeded and cultured at 1×10^5 cells/mL overnight in 6-well plates on cell climbing slices. Cells were then treated with different 5-HMF concentrations (0.1 IC_{50} and IC_{50}) or C48/80 (15 μg/mL). After stimulation for 1 h, cells were washed twice with pre-warmed (37 °C) 1 × PBS (Solarbio), before being fixed in 4 % formaldehyde solution in PBS at room temperature for 10 min. Then cells were washed three times in PBS. Afterwards, cells were permeabilized in 0.5 % Triton X-100 solution (Sigma) for 5 min at room temperature, and again washed three times in PBS. They were then stained in TRITC Phalloidin (100 nM, Solarbio) at room temperature for 30 min in the dark. After this, slices were washed three times in PBS for 5 min each time, then placed upside down on a Fluoromount-GTM slide containing a drop of DAPI (10 μg/mL, Solarbio). The slices were sealed using transparent nail polish. Finally, cytoskeleton structures were examined on a fluorescence inverted microscope (Carl Zeiss), equipped with a TRITC excitation/emission filter (Ex/Em = 540/570 nm), and DAPI excitation/emission filter (Ex/Em = 364/454 nm). Image-Pro Plus was used to process images and add scale bars.

2.10. Western blot analysis

RBL-2H3 and P815 cells were seeded and cultured at 1×10^5 cells/mL overnight in 6-well plates. Plates were then washed twice in advanced Tyrode's solution to remove residual media. C48/80 (15 μg/mL) or different concentrations of 5-HMF (0.1 IC_{50} and IC_{50}) were administered to cells. After stimulation for 1 h, cells were lysed in 100 μL radio-immunoprecipitation assay lysis buffer (RIPA; Sigma). Protein concentrations were measured using a BCA protein assay kit (Invitrogen, Grand Island, NY, USA). Western blot analyses were performed according to previous methods. Briefly, samples were boiled for 5 min, and equal quantities of protein (20 μg/well) were analyzed on a 10 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE, Solarbio). After electrophoresis, separated proteins were transferred to a polyvinylidene fluoride membrane (Thermo Fisher Scientific, USA). The membrane was blocked for 1 h at room temperature in tris-buffered saline (TBS) containing 5 % fat-free dried milk (BD, MD, USA). Next, membranes were incubated overnight at 4 °C with different antibodies (p-Lyn, Lyn, and Syk were all purchased from Abcam of USA; p-Syk was purchased from Thermo Fisher Scientific; p-p38, p38, p-JNK and JNK were purchased from Cell Signaling Technology) at a 1:1000 dilution as suggested by manufacturers. After washing three times in TBS containing Tween-20 (Solarbio), blots were further incubated with anti-rabbit IgG-horseradish peroxidase-conjugate antibody (Abcam) at a 1:2000 dilution for 1 h at room temperature. After secondary antibody addition, membranes were developed using an enhanced chemiluminescence detection kit (Invitrogen) and a model LAS-4000 luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan). For densitometric analyses, image J was employed.

2.11. Statistical analysis

All data were presented as the mean ± standard deviation (SD) of three independent experiments, performed in triplicate. All data were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's tests. Graph Pad Prism® software (GraphPad Software, La Jolla, CA, USA) was used to generate graphs and statistical analyses. * indicated statistically significant differences with control groups, $*P < 0.05$; $**P < 0.01$, $***P < 0.001$.

3. Results

3.1. 5-HMF induces anaphylactoid responses in mice and rats

We investigated anaphylactoid effects by injecting 5-HMF or C48/80 (positive control) into mice, through the caudal vein, accompanied by 0.4 % Evans blue dye. In terms of ear penetration, the control group did not show blue staining (Fig. 2A). In contrast, both 5-HMF and C48/80 groups exhibited increased blood vascular permeability compared with control group (Fig. 2B, C, D). Blue staining also showed a dose-dependent increase, *i.e.* when compared with the 5-HMF-L group, the severity of anaphylactoid effects in the 5-HMF-H group was more pronounced, $P < 0.001$ when stimulated with 5-HMF (Fig. 2C, D, E). ELISA assays of BN rat serum revealed that 5-HMF increased both His (Fig. 3A). and Gpx-1 (Fig. 3B) expression *in vivo*, but not dose-dependently.

3.2. The effects of 5-HMF on RBL-2H3 and P815 cell viability

Cell viability was assessed by MTT assay. Inhibition rate curves revealed the effects of different 5-HMF concentrations (0.1 IC_{50} and IC_{50}) on cell proliferation (Fig. 4). The IC_{50} of 5-HMF in RBL-2H3 and P815 cells was 2.01 mM and 5.17 mM, respectively. No effects on cell survival rates were observed after 5-HMF was applied for 1 h, therefore the IC_{50} was considered as the high-dose (5-HMF-H), and the 0.1 IC_{50} was considered as the low-dose (5-HMF-L) for subsequent experiments (Fig. 5).

3.3. The effects of 5-HMF on RBL-2H3 and P815 cell degranulation levels

Anaphylactoid reactions are accompanied by significant increases in β-Hex and His levels, which are both key degranulation markers [24–27]. To investigate anaphylactoid mechanisms induced by 5-HMF, these indicators were measured (Fig. 6). When compared with control cells, β-Hex and His levels in 5-HMF-administered cells were significantly increased, suggesting that 5-HMF induced cell degranulation *in vitro*.

3.4. The effects of 5-HMF on cytokine levels in RBL-2H3 and P815 cell supernatants

IL-4 and IL-6 are multifunctional cytokines that regulate immune responses [28,29]. The role of IL-6 is particularly critical in acute phase

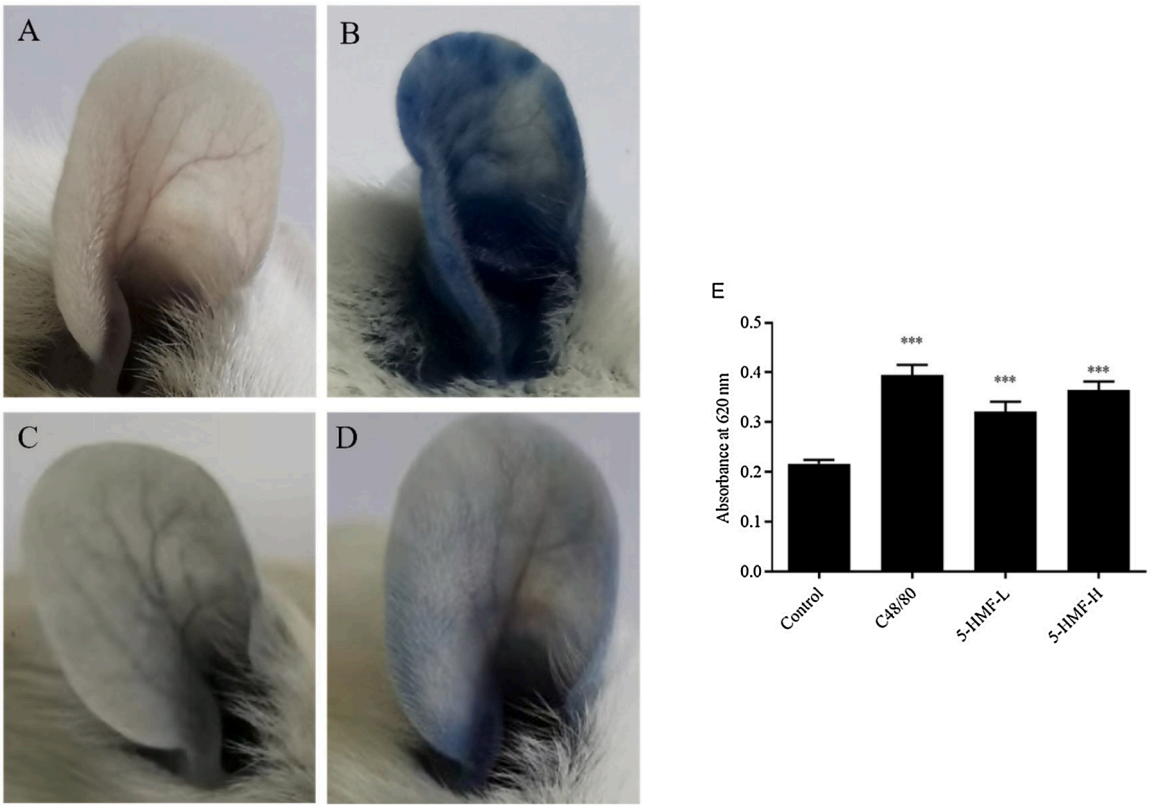

Fig. 2. The anaphylactoid effects of 5-HMF in mice. Data shown are the mean ± SD of three independent experiments. (A) Control, (B) C48/80 (25 mg/kg), (C) 5-HMF-L (7 mg/kg), (D) 5-HMF-H (35 mg/kg). (E) The absorbance at 620 nm of each group. An * indicates statistically significant differences when compared with the control group, ***$P < 0.001$.

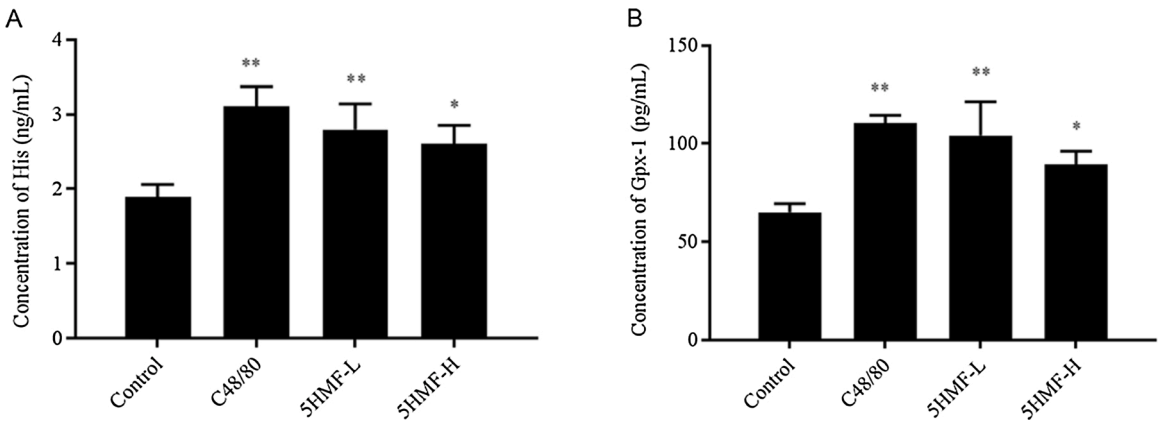

Fig. 3. The anaphylactoid effects of 5-HMF in BN rats. Data shown are the mean ± SD of three independent experiments. ELISA data represent His (A) and Gpx-1 (B) levels in BN rat serum. Doses of C48/80, 5-HMF-L and 5-HMF-H were 5 mg/kg, 5 mg/kg and 25 mg/kg, respectively. An * indicates statistically significant differences when compared with the control group, *$P < 0.05$, **$P < 0.01$.

reactions [29]. Our ELISA data revealed that 5-HMF dose-dependentlyincreased IL-4 and IL-6 expression levels in both cell lines (Fig. 7). Based on these data, 5-HMF induces *in vitro* anaphylactoid reactions in RBL-2H3 and P815 cells.

3.5. The effects of 5-HMF on RBL-2H3 and P815 cell morphology

5-HMF-induced degranulation morphology in RBL-2H3 and P815 cells was observed by toluidine blue staining (Fig. 8). Cells incubated with 5-HMF exhibited irregular morphology, with the extracellular release of blue granules (shown by red arrows). This trait is a main feature of cell degranulation [30]. Cytoskeletal rearrangement is also a key step in the degranulation process [30]. Phalloidin binds to filamentous actin (F-actin) with a high affinity [22,31], thus, the stain was used as a fluorescence probe to investigate cellular actin arrangement. We observed F-actin alterations in RBL-2H3 and P815 cells stimulated by 5-HMF (Fig. 9). Our data showed that the cellular cytoskeleton was altered; cell membranes were not intact and cytoskeletal changes became more aggravated as the 5-HMF dose increased. Some cytoskeletons ruptured, the edges of cells were destroyed and nuclei were exposed. These data indicated that 5-HMF induced *in vitro* anaphylactoid responses by promoting cytoskeleton rearrangements and cellular degranulation.

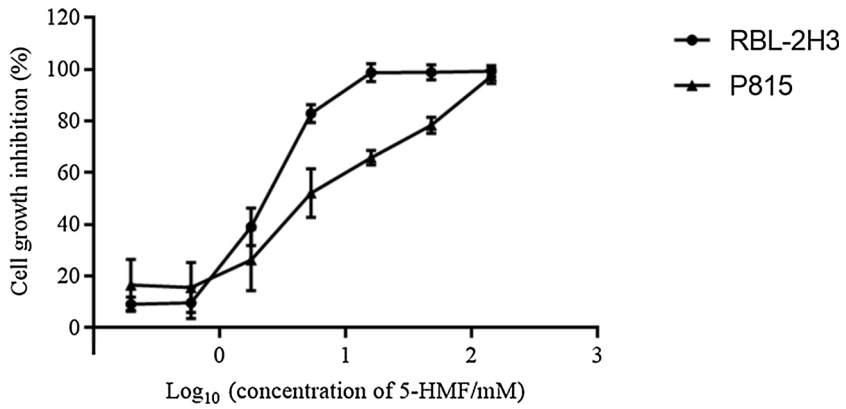

Fig. 4. Cell growth inhibition curves. Growth inhibition curves of 5-HMF in RBL-2H3 and P815 cells. Data are shown as the mean ± SD of three independent experiments.

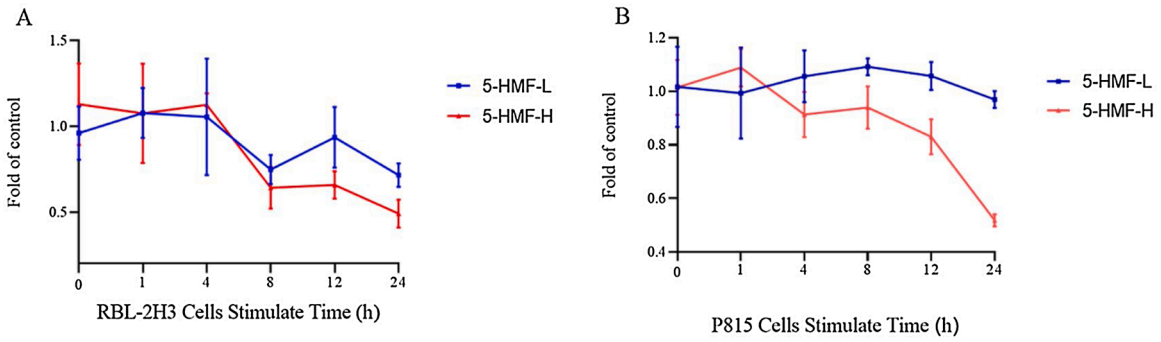

Fig. 5. Cell survival rates. Cell survival rates of RBL-2H3 (A) and P815 cells (B) stimulated with 5-HMF at different time points. Data are shown as the mean ± SD of three independent experiments.

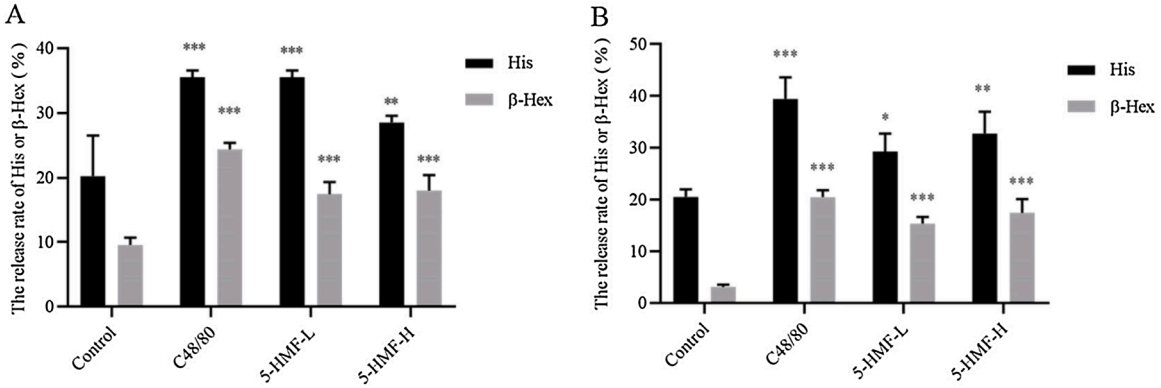

Fig. 6. His and β-Hex release rates from cells. 5-HMF promoted increases in β-Hex and His in RBL-2H3 (A) and P815 cells (B). Data are shown as the mean ± SD of three independent experiments. * indicates statistically significant differences when compared with control cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.6. The effects of 5-HMF on cell signaling in RBL-2H3 and P815 cells

Lyn activates Syk tyrosine kinase, and in turn Syk phosphorylates several signaling proteins, including the phosphorylated mitogen-activated protein kinases (MAPKs), such as p-p38 and p-JNK, increasing cell degranulation and cytokine release [32,27]. Thus, we investigated 5-HMF effects on these signaling pathway in RBL-2H3 (Fig. 10) and P815 cells (Fig. 11). We observed that 5-HMF stimulated increased phosphorylation of Lyn and Syk. Furthermore, 5-HMF upregulated p-p38 and p-JNK levels. These data indicated that 5-HMF induced anaphylactic reactions by upregulating the phosphorylation of Lyn, Syk, MAPKs in a dose-dependent manner.

4. Discussion

It is accepted that allergic diseases exert negative impacts on life. Anaphylactoid reactions are similar to type I hypersensitivity reactions, which are due to particular drug and food adverse reactions [33]. Although anaphylaxis and anaphylactoid reactions may be difficult to distinguish clinically as mast cells and basophildegranulation occur in both conditions, they involve different mechanisms [34]. During the anaphylactoid cascade, mast cells and basophils play pivotal roles, and are responsible for the rapid release of mediators such as His, β-Hex and cytokines [35,24,25,27]. Similar phenomenon also has been observed in the process of anaphylactoid reactions induced by paracetamol, aspirin

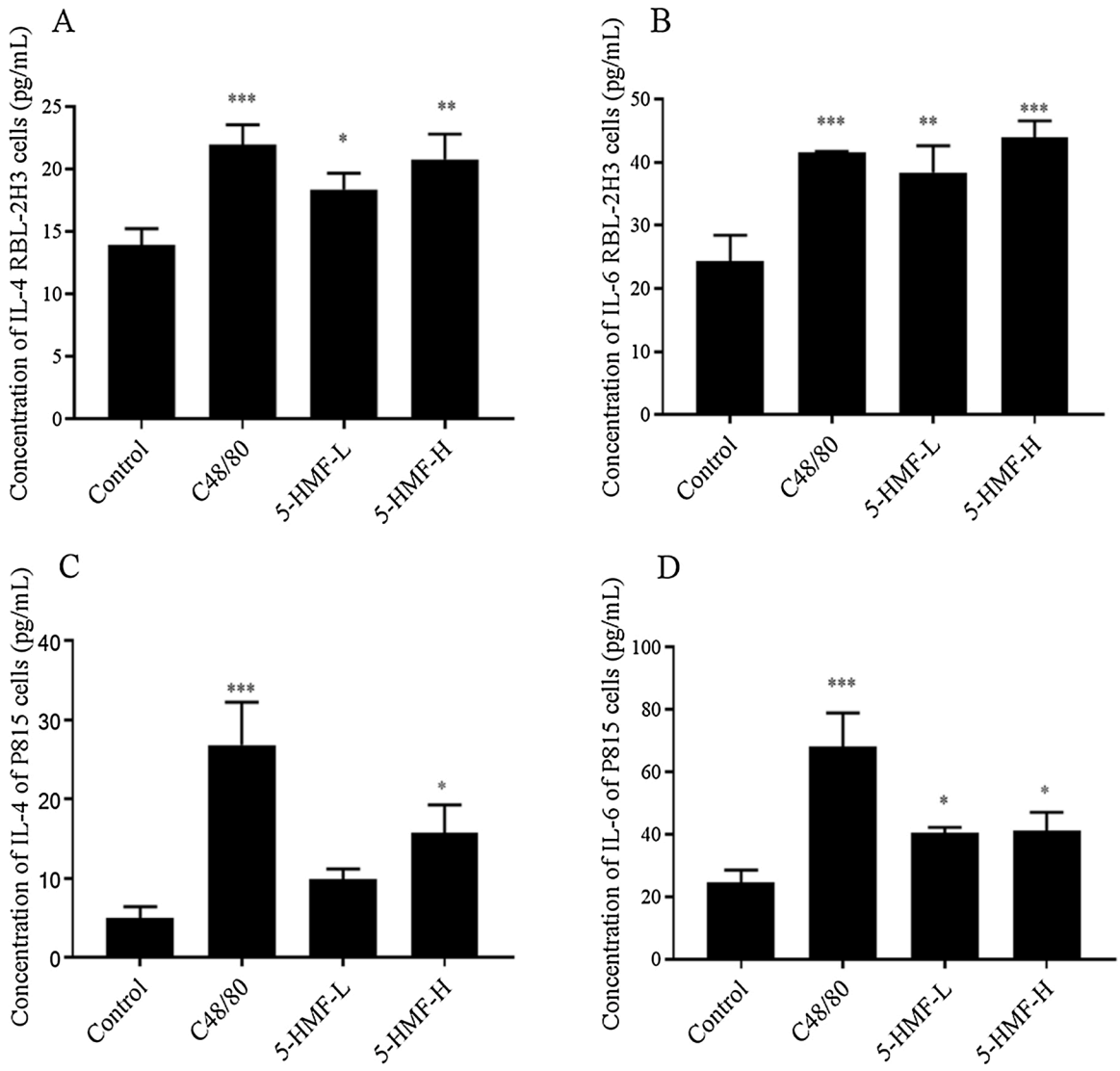

Fig. 7. IL-4 and IL-6 levels in cells. 5-HMF increased IL-4 and IL-6 expression levels in supernatants from RBL-2H3 and P815 cells. Data are shown the as mean ± SD of three independent experiments. (A) RBL-2H3, IL-4, (B) RBL-2H3, IL-6; (C) P815, IL-4, (D) P815, IL-6. * indicates statistically significant differences when compared with control cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and other non steroidal anti-inflammatory drugs [10,34]. C48/80 is a common stimulus that stimulates anaphylactoid reactions by releasing mediators from RBL-2H3 and P815 cells [35,24,25]. Due to increasing anaphylactic incidences [14,13], studies based on regulatory mechanisms underpinning the condition must be increased.

Our study confirmed *in vivo* and *in vitro* anaphylactoid effects of 5-HMF. In *in vivo* analyses, blood vessel permeability was enhanced by C48/80 and 5-HMF. ELISA analyses of rat serum revealed that 5-HMF upregulated both His and Gpx-1 levels. We also observed an interesting phenomenon during rat marker measurements. Although His and Gpx-1 levels increased significantly, there were no dosing effects. The levels of His and Gpx-1 in the 5-HMF-L group were higher than the 5-HMF-H group. This may be because 5-HMF at a high dose produces more eosinophils, reducing His and Gpx-1 levels, as eosinophils appear to enhance immune responses in rats [36].

For *in vitro* work, anaphylactoid cell models, based on RBL-2H3 and P815 cell lines were established. For anaphylactoid analyses, P815 and RBL-2H3 cells are more advantageous [37]. P815 cells are mouse mastocytoma cells, which contain only two γ subunits of the Ig E high affinity receptor, but do not contain the α and β subunits [38]. The α subunit is the binding section of Ig E and the receptor. It can play a role only when it is combined with the α subunit. The β subunit is not necessary. The γ subunit is responsible for the conduction of signal pathways [39]. Therefore, P815 cells are used for anaphylactoid research. The carrier(P815 cells) effectively avoids the interference of the IgE high-affinity receptor, FcεRI on the surface of mast cells. RBL-2H3 cells express high levels of the IgE high-affinity receptor on their surface, and can be used to establish type I hypersensitivity models, suitable for anaphylactoid model research. 5-HMF substantially altered RBL-2H3 and P815 cell morphology, promoting degranulation and destroying cytoskeletal structures. 5-HMF significantly increased His and β-Hex release, and upregulated IL-4 and IL-6 production. Toluidine blue staining also revealed degranulation effects. The degranulation pathway moves granules from the cell interior towards the plasma membrane [24,25]. This is followed by cortical actin depolymerization, regulated by the coronins, coro1A and coro1B [40]. Coronins are highly conserved actin-binding proteins located in the frontal regions of motile cells [41]. Actin fibers, also known as microfilaments (MF), are spiral fibers composed of cytoskeleton actin, with a diameter of 5–7 nm [42]. Therefore, we also assessed the cytoskeleton of RBL-2H3 and P815 cells, before and after drug stimulation. Our data showed that the cytoskeleton in both cell lines were incomplete after drug stimulation. These

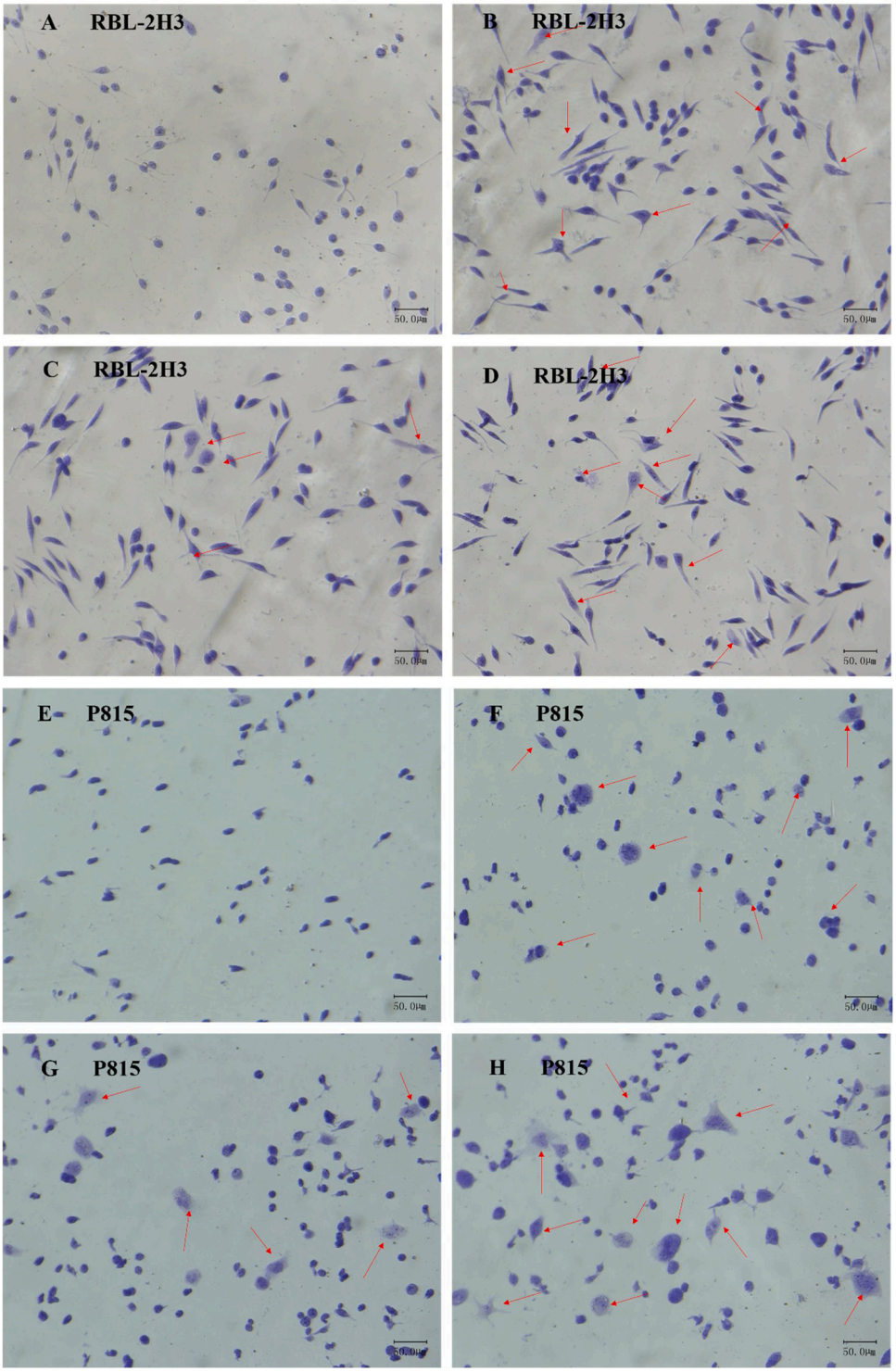

Fig. 8. The effects of 5-HMF on RBL-2H3 and P815 cell degranulation. (A) RBL-2H3, control, (B) RBL-2H3, C48/80, (C) RBL-2H3, 5-HMF-L, (D) RBL-2H3, 5-HMF-H, (E) P815, control, (F) P815, C48/80, (G) P815, 5-HMF-L, (H) P815, 5-HMF-H. Cells with adjacent red arrows exhibit degranulation. Scale bar = 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

results reflected the severity of anaphylactoid responses induced by 5-HMF.

We observed that 5-HMF promoted Lyn, Syk, p38, and JNK protein phosphorylation. From previous studies, Lyn and Syk are crucial molecules implicated in inflammatory responses [43,44]. Lyn and Syk are involved in signal transduction pathways triggered by the allergen-mediated activation of immune receptors in inflammatory cells [43,44], *i.e.*, 5-HMF induced degranulation of RBL-2H3 and P815 cells by up-regulating Lyn and Syk phosphorylation levels. In addition to degranulation, 5-HMF was also implicated in cytokine upregulation, which may due to its influence on p-p38 and p-JNK expression.

5-HMF is a product of the Maillard reaction, which is a non-enzymatic reaction between reducing sugars and amino acid compounds [45]. During the thermal processing and storage of saccharide-containing drugs and food, glycosylation products such as 5-HMF are generated [7]. These can occur in traditional Chinese

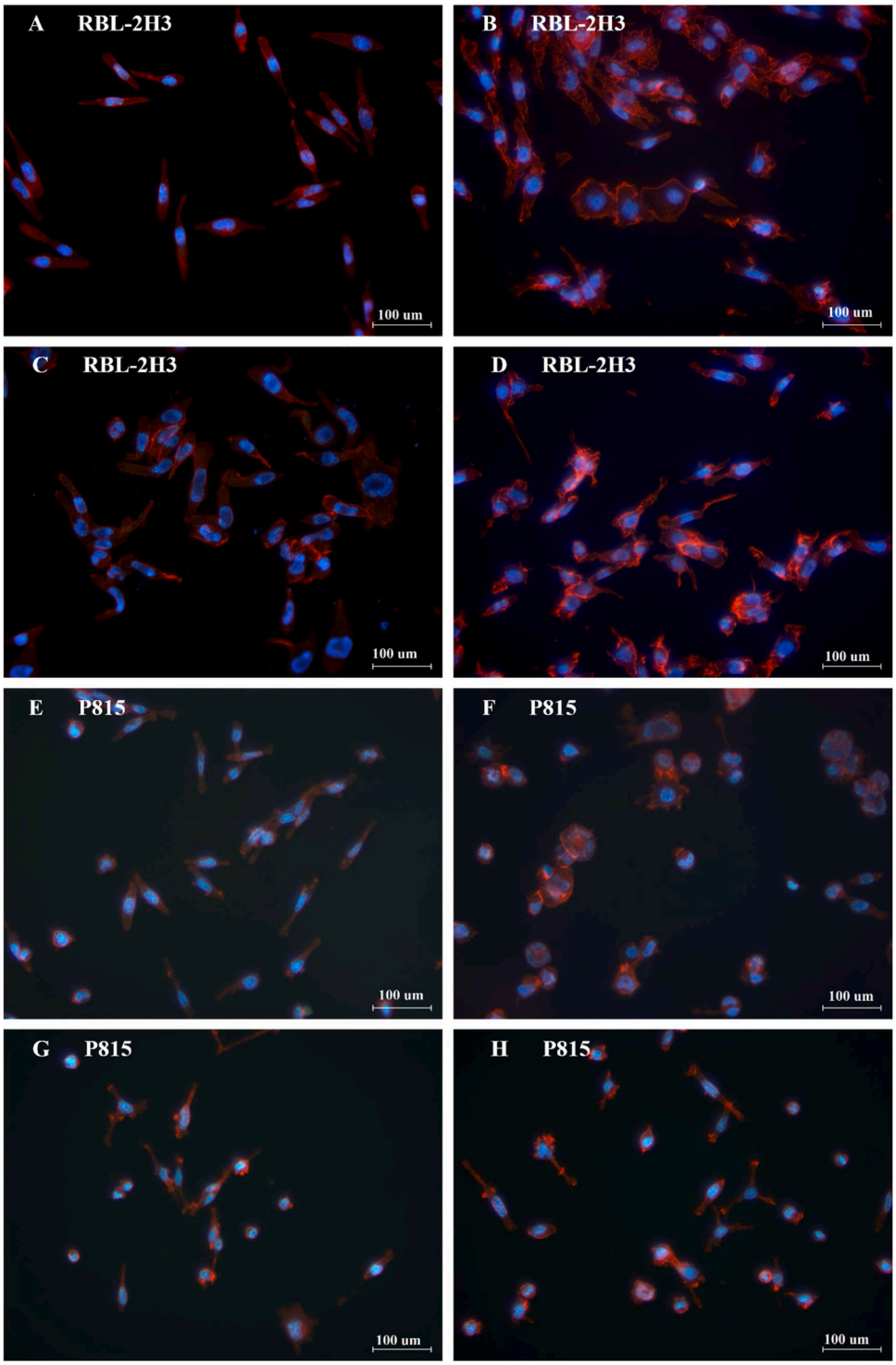

Fig. 9. The effects of 5-HMF on RBL-2H3 and P815 cytoskeletons. (A) RBL-2H3, control, (B) RBL-2H3, C48/80, (C) RBL-2H3, 5-HMF-L, (D) RBL-2H3, 5-HMF-H, (E) P815, control, (F) P815, C48/80, (G) P815, 5-HMF-L, (H) P815, 5-HMF-H. Scale bar = 100 μm.

medicine injections, dried fruit, bread, coffee, *etc.* [46]. This possesses a significant safety threat to pharmaceutical preparations and foods containing saccharides.

Our previous study showed that 5-HMF had immune-sensitizing potential [7]. The results of this study showed that 5-HMF induced anaphylactic reactions *in vivo* by elevating His and Gpx-1 levels in rats. Moreover, 5-HMF evoked β-Hex release, and the secretion of IL-4 and IL-6 from both RBL-2H3 and P815 cells, activating mast cell/basophil degranulation in the process. We further elucidated that Lyn, Syk, and MAPK phosphorylation was involved in 5-HMF-induced anaphylactic reactions *in vitro*. In view of these data, recent research has shown that 5-HMF causes anaphylactic symptoms by acting as a H_1 receptor agonist [46].Our study has begun in 2016 [7], the findings by He etc. and our group are mutually supportive and complementary. This observation potentially illustrates why 5-HMF increases vascular permeability in ICR mice. These data suggest that a single intravenous exposure to 5-HMF represents a severe safety issue, therefore more research is required to control and monitor 5-HMF levels in foods, and formulate regulatory

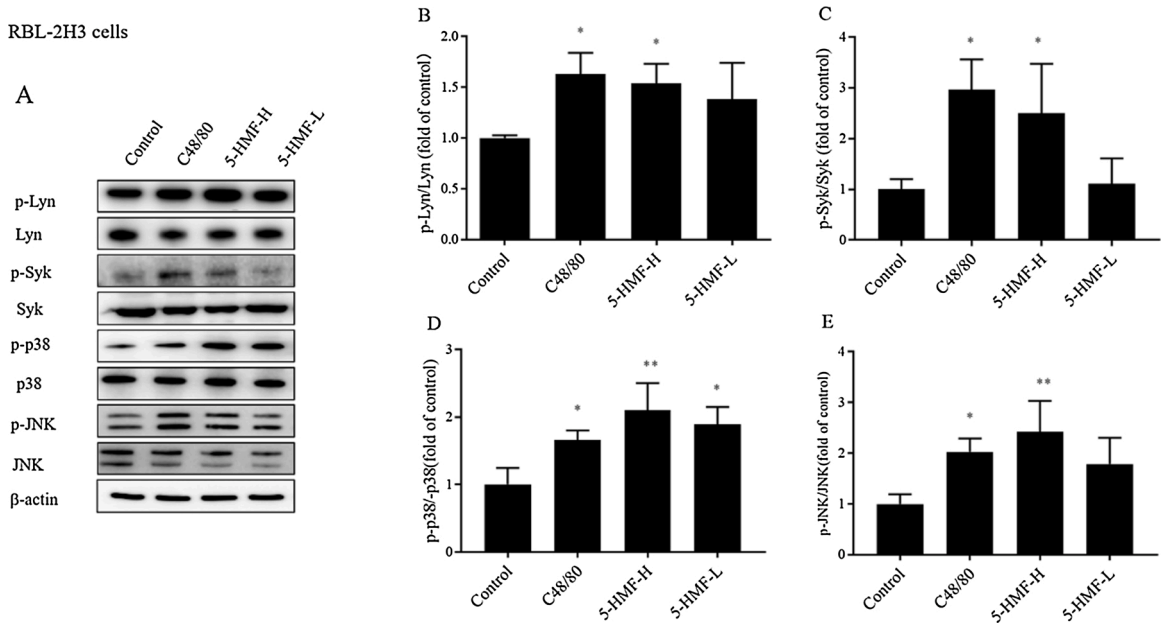

Fig. 10. The effects of 5-HMF on signaling events in RBL-2H3 cells. Western blots (A). Data are shown as the mean ± SD of three independent experiments. (B) RBL-2H3, p-Lyn/Lyn, (C) RBL-2H3, p-Syk/Syk, (D) RBL-2H3, p-p38/p38, (E) RBL-2H3, p-JNK/JNK. * indicates statistically significant differences when compared with control cells. *$P < 0.05$, **$P < 0.01$.

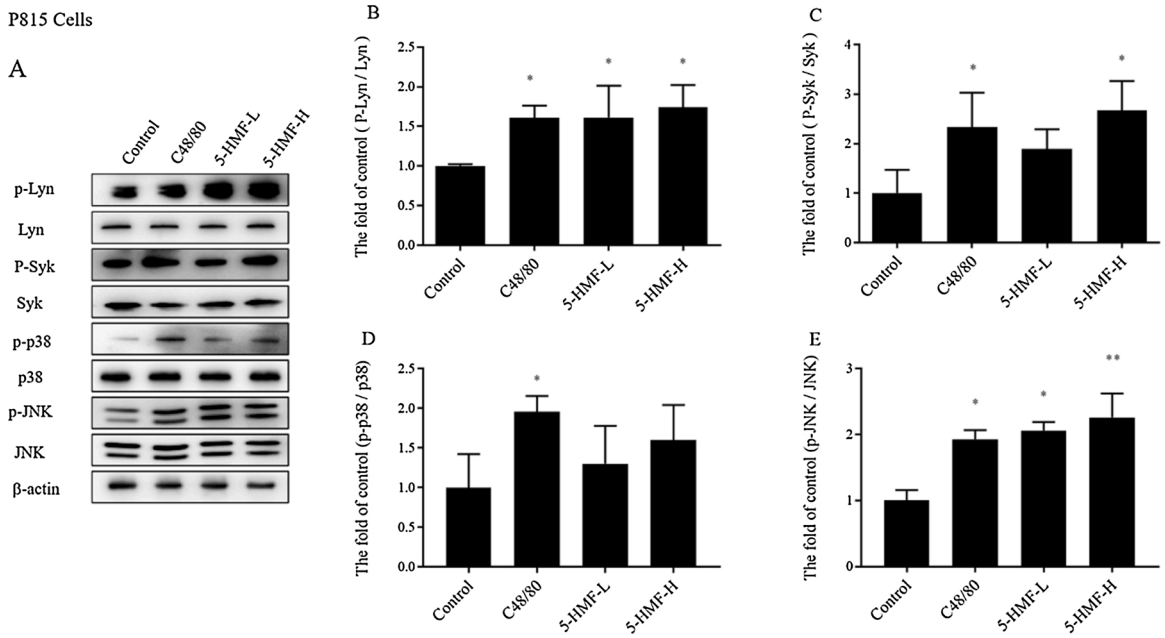

Fig. 11. The effect of 5-HMF on signaling events in P815 cells. Western blots (A). Data are shown as the mean ± SD of three independent experiments. (B) P815, p-Lyn/Lyn, (C) P815, p-Syk/Syk, (D) P815, p-p38/p38, (E) P815, p-JNK/JNK. * indicates statistically significant differences when compared with control cells. *$P < 0.05$, **$P < 0.01$.

5-HMF limit levels across pharmaceutical and food industries.

5. Conclusions

Based on our findings, 5-HMF induces anaphylactic reactions both *in vivo* and *in vitro*. The promotion of 5-HMF safety limits may improve safety in clinical drug use and food industry settings.

Authorship contributions

Hongtao Jin, Ni Lin, Sheng Lin, Jiuming He, Qingfen Zhu: Experimental guidance and editing manuscript; Encan Li, Ni Lin: writing manuscript and operating experiments; Ruirui Hao, Linlin, conducting cell experiments and data analysis, Xiaoyu Fan, Guang Hu, conducting animal experiments and data analysis.

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Declaration of Competing Interest

The authors report no declarations of interest.

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